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PROTECTIVE ROLE OF XINNAONING TABLET IN ISCHEMIC STROKE IN RAT MODEL

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Abstract

Background: Stroke has been considered as the second leading cause of death worldwide. The survivors of stroke experience different level of impair brain function. In China, Chinese traditional medicine had been widely accepted for stroke therapy and prevention. In this study, we developed Traditional Chinese Medicine based *Xinnaonin*g (peace of heart and brain) Tablet and tested its protective role for ischemic stroke in rat model.
Material and Methods: Male Wistar rats (n=60) with 12 weeks old and weight from 180 to 200 gram were randomly divided to five groups (n=12). For the groups with Xinaoning administration, the drug was administrated to rats once per day for 7 consecutive days. The blood clotting time and the thrombus wet weight was measured. Serum samples were collected from each rat for further Measurement of biochemical indicators.
Results: Our results demonstrated that *Xinnaoning* tablet reduced lactate acid (LD) level and increased lactic acid dehydrogenase (LDH) in cerebral ischemia model as well as reduced the infarct size caused by stroke. Besides, evaluation of the level of different ATPases suggested *Xinnaoning* tablet could modulate ATPases activity and confer a protective role in brain. Moreover, analysis indicated Xinnaoning tablet have the anti-coagulation effect *in vivo* which may contribute to the protection of ischemia.
Conclusion: Our findings suggest that Xinnaoning tablet may be a potential way for cerebral ischemia prevention.

Keywords: Traditional Chinese medicine, Xinnaoning, ischemic stroke, anti-coagulation effect

Introduction

Stroke, sometimes referred as cerebrovascular accident (CVA), or cerebrovascular insult (CVI), is a disease of loss of brain function due to the changing of blood supply in brain and resulting in neuron death (Flynn et al., 2008;Sims and Muyderman, 2010). The syndrome of stroke includes suddenly experienced paralysis, impaired speech ability or loss of vision (Moskowitz et al., 2010). The stroke can be classified into two major types: ischemia (lacking of blood flow) or hemorrhage(bleeding) (Sims and Muyderman, 2010). Except high mortality, stroke is the primary cause of disability in adult (Doyle et al., 2008). The survivors of stroke generally experience extremely hard condition, such as loss of the ability to work or needing for self-care, which leads to a huge burden to both survivors and their families (Moskowitz et al., 2010).

Current treatment for ischemic stroke is limited due to the damage for neuron in adults is considered as permanent and the adult nervous system is incapable for regeneration. Recently, researchers starts to realize that adult nervous system exhibiting focal areas of ongoing neurogenesis and stem cell transplantation may be an alternative way for neuron regeneration (Moskowitz et al., 2010). However, these speculations are still far from clinical application. Therefore, prevention of ischemic stroke in individuals with high risk is recommended.
The pathobiology and mechanism of stroke is extremely complicated and less understood. The cerebral ischemia (ischemic stroke), which is the major type of stroke, accounts for more than 85% stroke cases (Flynn et al., 2008;Sun et al., 2010). Currently, factors such as the family history of cerebrovascular diseases, age, sex (Hankey, 2006;Allen and Bayraktutan, 2008), hypertension (Lawes et al., 2004a), diabetes (Lawes et al., 2004b), hypercholesterolemia (Amarenco et al., 2006) as

well as cigarette smoking (Bonita et al., 1999), which are often existed together, have been considered to contribute for occurrence of 60-80% ischemic stroke in normal populations (Hankey, 2006;Allen and Bayraktutan, 2008).

Generally, in ischemic stroke, blood supply to part of the brain is decreased due to thrombosis, embolism, systemic hypoperfusion and venous thrombosis (Shuaib and Hachinski, 1991;Stam, 2005;Donnan et al., 2008). Therefore, oral administration of anticoagulants such as warfarin or aspirin had been widely used for stroke prevention for a longtime, especially for preventing the recurrence (secondary prevention) after a stroke or transient ischemic attack (Saxena and Koudstaal, 2004). However, long term administration of aspirin also demonstrated side effect such as gastrointestinal bleeding. On the other hand, alternative anticoagulants drug such as thienopyridines caused less bleeding but more expensive (Hankey et al., 2000). Therefore, seeking for affordable medicine with fewer side effects to prevent ischemic stroke is more beneficial.

In the past decade, application of traditional Chinese Medicine (TCM) for stroke prevention and recovery has increased drastically (Gong and Sucher, 2002;Junhua et al., 2009;Liao et al., 2012). In China, more than 100 traditional medicines have been used for treating stroke and some of their therapeutic effect have been confirmed by clinical studies (Gong and Sucher, 2002). However, the mechanism for those TCM based medicine is still illusive. In this study, we developed TCM based Xinnaoning (peace of heart and brain) tablet and tested it protective role for ischemic stroke in rat model. Our results demonstrated that Xinnaoning tablet could reduce lactate acid (LD) level and increase lactic acid dehydrogenase (LDH) in cerebral ischemia model. Further analysis indicated Xinnaoning tablet have that anti-coagulation effect *in vivo*. In conclusion, our data suggested that Xinnaoning tablet may be a potential way for cerebral ischemia prevention.

Materials and Methods

Ethics statement

The animal procedures were approved by the 371st Center Hospital of People's Liberty Army in accordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China). All surgery was performed according to recommendations proposed by the 371st Center Hospital of People's Liberty Army, and all efforts were made to minimize suffering. Animals were housed in a temperature-controlled room with proper darkness-light cycles, fed with a regular diet, and maintained under the care of the Experimental Animal Center of the 371 Hospital of People's Liberty Army.

Preparation of Xinnaoning tablet and suspension

Xinnaoning (Peace of heart and Brain) tablet composes of Ginseng (root of *Panax trifolius*), Angelica, Ligusticum chuanxiong Hort and Astragalus membranaceus. Briefly, 130 g Ginseng, 380 g Astragalus membranaceus, 500 g Angelica and 500 g Ligusticum chuanxiong Hort were grinded to 10-mesh powder and mixed together. The powder mixture was boiled twice with 8 folds distilled water for two hours to get crude extract. The liquid extract was filtered and vaporized under 80°C until the relative density reaching 1.15 to 1.30. Then 120 g Ginseng and Astragalus membranaceus was grinded to 100-mesh powder and the powder was added to liquid extract along with starch. The mixed powder-liquid extract was dried and grinded to 14-mesh crude powder again. Magnesium steam rate was added to the crude powder with a V/V ratio of 1 to 100, followed by pressing of tablet press machine for getting tablet (0.3 g for each). The quality control criteria of herbs used for Xinnaoning followed the instruction of *Chinese Pharmacopoeia* (2010 version). The Xinnaoning suspension was used for gavage of rats. Rats in experiment groups were administrated orally with three different dosages (75 mg/mL for high dose, 37.5 mg/mL for medium dose and 18.8 mg/mL for low dose).

Animals and samples

For ischemia rat model, male Wistar rats (n=60) with 12 weeks old and weight from 180 to 200 gram were purchased from Animal Centers of Hebei Province and used in this study. All rats were randomly divided to five groups (n=12). For the groups with Xinaoning administration, the drug was administrated to rats once per day for 7 consecutive days. Starting from the sixth day of Xinnaoning administration, only water was used to feed rats. In the seventh days, one hour after administration of Xinnaoning, the focal cerebral ischemia model by endovascular suture occlusion was conducted as previously described (Uluc et al., 2011). Then the brain tissue of rats were removed and homogenized with PBS at a ratio of 1 to 9 for further tests.

To measure the anti-coagulation effect of Xinnaoning *in vivo*, male Ischemic stroke rats (n=60) were randomly divided to five groups as well. 3 groups of rats were administered with Xinaoning suspension with different dose (high, medium, low) as above. One group of rats with administration of aspirin (Sigma-Aldrich) orally and another untreated group were included as controls. The blood clotting time and thrombus wet weight were measured as previously described (Fredrich et al., 1994;Fukuda et al., 2006). Serum samples were collected from each rat for further experiment.

Histology analysis for the brain tissue

To measure the infarct size of brain tissue, additional 10 rats were randomly divided to control groups and Xinnaoning group(n=5). High dose of Xinnaoning was administered to rats as mentioned above. Rats was anaesthetized and fixed with injection of 4% paraformaldehyde. The brain tissue was removed and fixed in 4% paraformaldehyde for another 72 hours before sampling. For the measurement of infarct size of brain tissue, the brain slices were sampled in an interval of 120um. Then the slices were treated as following sequence: air dry for 15min, 95% ethanol for 15min, 70% ethanol for 1min, 50% ethanol for 1min, ddH2O 1min for twice, cresol purple for 10min, ddH2O for 1min, 50% ethanol for 1min, 70% ethanol for 2min, 95% ethanol for 2min, 100% ethanol for 2min, xylene for 10min. After all the procedures, the slice was sealed by neutral balsam for microscope. The infarct size of sample was captured and calculated by Image J software.

Measurement of biochemical indicators

The total protein level, lactate acid (LD) level and lactic acid dehydrogenase (LDH) level were measured by using commercial kits from Nanjing Jiancheng Biotech (Nanjing, Jiangsu, China) according to manufacturer's instruction with an ultraviolet spectrophotometer (UV-2000, Unico Instrument, Shanghai, China). Measurement of Na+-K+-ATPase, Mg++-ATPase and Ca++-ATPase were conducted with commercial kits from Nanjing Jiancheng Biotech according manufacturer's instruction as well. Prothrombin concentration of was measured by using a rat prothrombin ELISA kits (Changyi Biotech, Shanghai, China).

Statistical analysis

Statistical analysis was performed by SPSS 19.0 software. Data are presented as Mean ± standard deviation (SD). A single factor pair-wise ANOVA statistical analysis was used to evaluate the significance in differences between levels of test parameters among indicated groups of rat. A two tailed *P*-value of less than 0.05 was considered significant.

Results

Xinnaoning tablet reduce lactate acid (LD) level and increase lactic acid dehydrogenase (LDH) in cerebral ischemia model

The Xinnaoning tablet was made based on the theory of TCM, the major intergradient were derived from Ginseng (root of *Panax trifolius*), Angelica and Ligusticum chuanxiong Hort. Reports have demonstrated that red ginseng extract pretreatment protect mice neuron cells from ischemia-induced oxidative stress and apoptosis(Cheon et al., 2013). Moreover, in 5-min ischemic gerbils using a step-down passive avoidance task and subsequent neuron and synapse counts in the hippocampal CA1 region, crude extract from Ginseng root prevents learning disability and neuronal loss(Wen et al., 1996). Furthermore, Angelica and Ligusticum chuanxiong Hort have been demonstrated to enrich blood, promote blood circulation and modulate the immune system as well(Wu and Hsieh, 2011). Therefore, we expected that combination of these herbs is able to improve the viability of heat and brain based on TCM.

To confirm our speculation, we first created the cerebral ischemia rats model from different groups by using methods as previously described (Uluc et al., 2011). Based on analysis the LD and LDH level in the brain tissue, the ischemia group demonstrated increased level of LD from 0.336 mmol/L to 0.49 mmol/L (**Table 1**), which suggesting the less oxygen supply for the brain tissue. On the other hand, we noted that the LDH level of ischemia group also decreased from 11812 u/grot to 7866 u/grot (**Table 1**). However, with the administration of Xinnaoning, the LD level was much lower than ischemia group, demonstrated a better viability of brain (**Table 1**). Moreover, the LDH level of groups administered with Xinnaoning were similar to MOCK group. Additionally, it appears that different dosage of Xinnaoning having minimum effect on brain LD level and LDH level. Taken together, these data suggesting the Xinnaoning could effectively reduce the LD level if oxygen supply is limited for brain.

Table 1. Affection of Xinnaoning for Brain LD and LDH level

Groups	LD level (mmol/l)	LDH (u/grot)
Control	0.336±0.042**	11812±2345**
Ischemia group	0.49±0.079	7866±1374
High dose	0.374±0.052**	10529±1518**
Medium dose	0.392±0.062**	9723±1257**
Low dose	0.374±0.035**	11135±1151**

Significant different had been marked as "**", which indicated that P<0.05.

Xinnaoning tablet reduce infarct size of the brain slice in treated rats

To further confirm the protective effect of Xinnaoning tablet, we also evaluated the infarct size in control groups and Xinnaoning treated groups, since dosage of Xinnaoning plays little roles, only brain slice from control rats and high dose Xinnaoning treated rats were compared. As demonstrated in Figure.1, in the control rats, almost one sixth of the brain slice formed a plaque which demonstrated the degradation and necrosis of neurons. However, Xinnaoning treatment significantly decreased the infarct size in the brain slice. Quantification of image data demonstrated the infarct size of the brain slice was reduced from 30mm^3 to 15mm^3, indicated a less damage caused by the stroke in brain tissue.

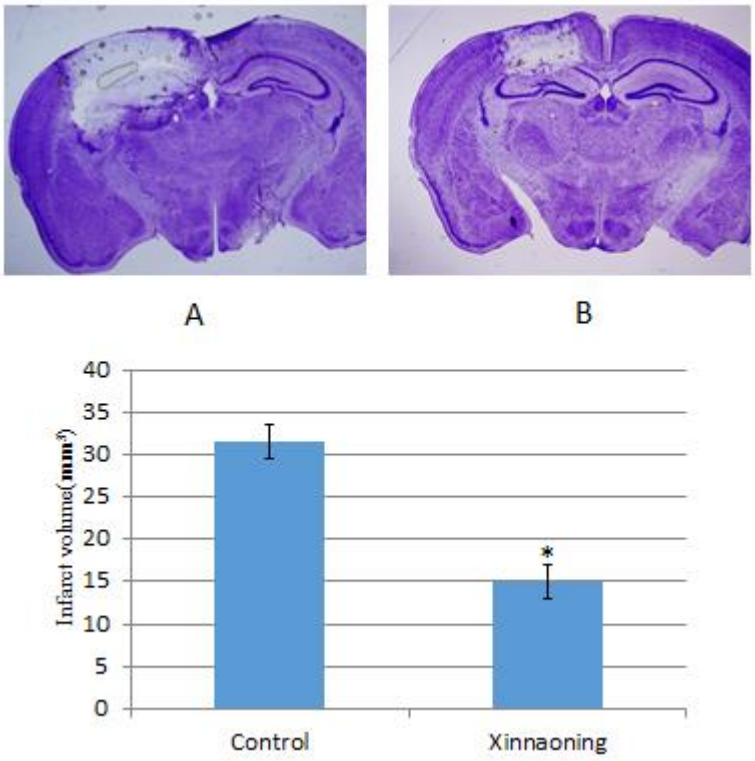

Figure 1. Xinnaoning tablet treatment in rats reduced the infarct size caused by ischemic stroke, A: The neuron degradation and necrosis was visualized by Nissl stain in stroke rats. B. Stroke rats with Xinnaoning treatment demonstrated reduced neuron degradation and necrosis. C. Quantification of the area of neuron degradation and necrosis between groups.

Effect of Xinnaoning on ATPase activity of brain

To further verified the protective role of Xinnaoning on the brain during the cerebral ischemia, we evaluated the level of different ATPases as well. Based on our data, by induction of cerebral ischemia, activity of all three ATPases, Na+-K+-ATPase, Mg++-ATPase and Ca++-ATPase were significantly reduced (**Table 2**). However, administration of Xinnaoning increased activities of all three ATPases, while dosage have little role to increase the ATPase activity for Na+-K+-ATPase and Ca++-ATPase (**Table 2**). Moreover, it is notable for Mg++-ATPase; the low dose of Xinnaoning demonstrated better improvement than medium dose and high dose (**Table 2**).

Table 2. Xinnaoning's effect on brain ATP level

Groups	Na^+-K^+-ATPase (μmolp/mgprot/hour)	Mg^{++}-ATPase (μmolp/mgprot/hour)	Ca^{++}-ATPase (μmolp/mgprot/hour)
Control	4.732±0.709**	3.048±0.553**	2.357±0.423**
Ischemic group	3.242±0.514	1.994±0.503	1.349±0.279
High dose	4.215±0.765**	2.478±0.411*	2.159±0.438**
Medium dose	4.173±0.74**	2.504±0.363*	2.176±0.802**
Low dose	4.072±0.425**	3.342±0.279**	1.894±0.378**

Significant different had been marked as "**", which indicated that P<0.05.

Xinnaoning has anti-coagulation effect

During the cerebral ischemia progression, oral administration of anticoagulation is recommended as a choice of therapy for both primary and secondary ischemic stroke prevention in patients with atrial fibrillation or any other risk factors (Lip and Lane, 2013;Toth, 2013). Therefore, it is interesting to know if Xinnaoning has any anti-coagulation effect which may benefit ischemic stroke prevention. As a result, both blood clotting time and thrombus wet weight of rats administrated with Xinnaoning were measured. The untreated rats and rats administered with Aspirin were included as negative and positive controls. Based on the result, negative control, Aspirin significantly reduced the thrombus wet weight and increased the clotting time of blood as expected (**Table 3**). Moreover, the prothrombin concentration was also evaluated in Aspirin group (positive control). On the other hand, the thrombus wet weight from groups administrated with different dose of Xinnaoning was similar but significantly lower than negative controls as well (**Table 3**). It is also notable that different dosage of Xinnaoning demonstrated minimum effect on clotting time and prothrombin concentration as well. Although administration of Xinnaoning extended the clotting time and increase the blood prothrombin concentration, these two indicators were still lower than Aspirin group (**Table 3**). Taken together, Xinnaoning deed demonstrates the anti-coagulation effect in rats may benefit for ischemic stroke prevention as well.

Table 3. The anti-coagulation effect of Xinnaoning *in vivo*

Groups	Thrombus wet weight (g)	Blood clotting time (s)	Prothrombin concentration
Control	0.036±0.006	105.8±43.6	0.097±0.118
Aspirin	0.020±0.003**	162.6±37.5**	0.424±0.436**
High dose	0.022±0.004**	146.9±39.3*	0.248±0.143**
Medium dose	0.024±0.004**	141.4±31.1*	0.180±0.233
Low dose	0.022±0.006**	143.9±47.3	0.196±0.169

Significant different had been marked as "**", which indicated that $P<0.05$

Discussion

The changing of blood supply in brain results in the loss of brain function and causing ischemic stroke (Flynn et al., 2008;Sims and Muyderman, 2010), especially the cerebral ischemic stroke accounts for more than 85% stroke cases and so it is a very common neurological disorder (Flynn et al., 2008;Sun et al., 2010). Therefore, prevention of ischemic stroke in individuals with high risk is recommended. In this study, we tested the Xinnaoning tablet; TCM herbs based medicine, for its protective role in cerebral ischemia. In our result, the LD level in Xinnaoning administrated rats was similar to the control groups. Although glucose is usually assumed to be the main energy source for living tissues, there are some evidence suggested that it is lactate, but not glucose, which is preferentially metabolized by neurons in the brain of several mammalian species such as mice, rats, and humans (Zilberter et al., 2010;Wyss et al., 2011). Therefore, reduced lactate acid level may not only suggest that less lactate acid been generated, but may imply more lactate acid had been metabolized for energy production and supporting brain in rats with Xinnaoning administration. However, it is an interesting question to further investigate that if Xinnaoning tablet could reduce lactate acid generation or lactate acid metabolism.

Our analysis of ATPases activities of Xinnaoning administered rats suggested a protective role on the brain as well. In ischemia stroke group, activities of all three different ATPases were significantly down-regulated suggesting a break of homeostasis in neuron cells with less oxygen supply. The Na+ATPase, was confirmed to control the cell volume which is important for homeostasis of neuron (Nylander-Koski et al., 2005). Na^+ATPase has specially role in neuron activity states, such as control and set the intrinsic activity mode of cerebellar Purkinje neurons (Forrest et al., 2012). Moreover, the plasma membrane Ca2+ATPaseare also important for the normal neuron function as it had been reported to regulating synaptic activity (Jensen et al., 2007). Therefore, the increased ATPases activities by administration of Xinnaoning prevent the break of homeostasis and demonstrated a protective role during the cerebral ischemia.

On the other hand, our data also suggesting that the anti-coagulation effect of Xinnaoning tablet was comparable to Aspirin. During the progression of cerebral ischemia, the undesired thrombosis or embolism due to the activation of coagulation system is considered as the major contributing factors (Shuaib and Hachinski, 1991;Stam, 2005;Donnan et al., 2008). Therefore, oral administration of aspirin or other anticoagulants is recommended for long term prevention of ischemic stroke (Saxena and Koudstaal, 2004). Therefore, administration of Xinnaoning may be an alternative way of aspirin for stroke prevention to avoid the side effect. Taken together, our data demonstrated a protective role of Xinnaoning tablet on brain during the cerebral ischemia and may be used for ischemic stroke prevention. However, its role in medical practice still needs further investigation in clinical trial.

Conflict of interest: The authors declare no conflicts of interest.

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